Suprabasal Spongiosis in Acute Eczematous Dermatitis: cFLIP Maintains Resistance of Basal Keratinocytes to T-Cell-Mediated Apoptosis

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In acute eczematous dermatitis, keratinocyte (KC) apoptosis caused by dermis-infiltrating, activated T cells plays a crucial pathogenetic role in the development of spongiosis, the histopathological hallmark of acute eczema. Remarkably, T-cell-mediated apoptosis of single KC, as well as spongiosis, is located predominantly in suprabasal epidermal layers, suggesting that antiapoptotic mechanisms protect basal KC. The cellular Flice-inhibitory protein (cFLIP) is known to block apoptotic CD95-signaling, and may therefore account for such a protection of basal KC. HaCaT KCs retrovirally transduced with the long form of cFLIP were effectively protected against T-cell-mediated apoptosis in KC monolayer/$CD4^+$ T-cell cocultures. *In situ* correlation of cFLIP protein expression and KC apoptosis in lesional eczematous skin showed a highly restricted expression of cFLIP in basal KC, whereas cleaved caspase-3 (as a surrogate marker of apoptosis) was detected predominantly in suprabasal epidermal layers. Thus, the modulation of the CD95 signaling pathway by the cell-intrinsic caspase-8 inhibitor cFLIP in basal KC may explain the spatial localization of spongiosis in suprabasal epidermal layers, and provides new insights into the pathogenesis of spongiosis formation in eczematous dermatitis.

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INTRODUCTION

Eczematous dermatitis is a T-cell-mediated inflammatory skin disease, where activated T cells may harm epidermal keratinocytes (KCs) by direct cell–cell contact or secreted proinflammatory cytokines, accounting for at least some of the features of spongiosis formation, the typical epidermal pathology of eczema (Trautmann *et al.*, 2000; Akiba *et al.*, 2002). We have previously shown that activated T cells infiltrating the skin induce apoptosis of single KC, as shown *in situ*, in the lesional skin of atopic dermatitis (AD) and allergic contact dermatitis (ACD). Interestingly, in these studies, apoptosis of single KC was detected predominantly in suprabasal epidermal layers, concomitant with suprabasal spongiosis formation. This spatial localization of KC apoptosis raises the question about the mechanism that might protect basal KC against T-cell-mediated apoptosis. Resting human KCs express low levels of CD95 that are upregulated by IFN-γ. In T-cell–KC cocultures, KCs undergo apoptosis induced by supernatants of activated T cells, direct contact with activated T cells, and artificial CD95 triggering through a soluble CD95 ligand (Trautmann *et al.*, 2000).

The CD95 signaling pathway is characterized by the sequential activation of caspases (Kumar, 2007). In this context, activation of the effector caspase-3 represents one of the key points in the transmission of the CD95 death signal leading to the biochemical and morphological changes of apoptosis (Lavrik *et al.*, 2005). The cellular Flice-inhibitory protein (cFLIP) blocks the CD95 signaling pathways through the inhibition of the initiator caspases-8/-10 within the death-inducing signaling complex, thereby preventing the activation of caspase-3 (Peter and Krammer, 2003), and was previously shown to be expressed in primary human KCs *in vitro* when compared with spontaneously transformed HaCaT KCs (Leverkus *et al.*, 2000).

In this study, we show that HaCaT KCs, ectopically expressing the long form of cFLIP ($cFLIP_L$) in the T-cell–KC coculture system, are protected against T-cell-mediated apoptosis. In biopsy specimens of acute eczematous dermatitis (AD/ACD), we found that cFLIP expression is highly restricted to the basal KC layer. Conversely, epidermal caspase-3 cleavage was almost exclusively detected in suprabasal epidermal layers close to areas of spongiosis. In contrast, expression of cFLIP in the basal epidermal layer could not protect KC from apoptosis in the biopsy specimen of lichen planus (LP), most likely through dominance of granzyme B positive cytotoxic effector T cells in the lichenoid infiltrate of this disease (Wenzel *et al.*, 2006).

In summary, these findings support the hypothesis that cFLIP may contribute to the resistance of basal KCs to

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Abbreviations: cFLIP, cellular Flice-inhibitory protein; KC, keratinocyte; LP, lichen planus

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death-receptor-induced, T-cell-mediated apoptosis in eczematous dermatitis, and provide an explanation for the spatial restriction of spongiosis to suprabasal epidermal layers.

RESULTS

T-cell-mediated keratinocyte apoptosis

In vitro, crystal violet stainings showed that incubation of HaCaT KCs with activated T cells or their supernatants, respectively, partially destroyed the HaCaT monolayer, mimicking spongiosis formation *in vivo* (Figure 1a). Activated T cells with direct contact to the HaCaT monolayer, as well as supernatants of activated T cells, induced significant HaCaT KC apoptosis compared with HaCaT cells cultured alone at day 2 (Figure 1b). Purified $CD4^+$ T cells were stimulated for 24 hours with a combination of anti-CD2/-CD3/-CD28 mAb, and then cells and supernatants were separately incubated with a blocking CD95L-specific mAb. In parallel, the sensitivity of cultured HaCaT KCs to CD95-mediated cell death was determined by crosslinking of CD95 with recombinant CD95L. As shown in Figure 1c, continuous CD95L blockade prevented cell death induced by activated T cells and supernatants to levels comparably achieved by the pan-caspase inhibitor z-VAD. Moreover, artificial CD95 crosslinking of HaCaT cells showed their sensitivity to CD95-mediated apoptosis. These findings show that under both coculture conditions, allowing or omitting direct cell–cell contact, HaCaT KC apoptosis induced by anti-CD2/-CD3/-CD28 mAb-activated T cells is mainly mediated through CD95L.

cFLIP inhibits T-cell-mediated apoptosis of keratinocytes

The intracellular protein cFLIP interferes with death ligand-induced apoptosis at the death-inducing signaling complex in KCs (Leverkus *et al.*, 2000; Wachter *et al.*, 2004). To evaluate the impact of upstream caspase inhibition in our experimental system, we first established $cFLIP_L$-expressing HaCaT KCs by retroviral transduction (Figure 2a and b and Figure S1, Supplementary Materials and Methods). Polyclonal $cFLIP_L$-expressing populations of HaCaT cells expressed comparable levels of CD95 as determined by flow cytometric analysis (Figure 2c), but were highly resistant to CD95-induced apoptosis when compared with control-infected cells (Figure 2d), indicating the functionality of ectopically expressed $cFLIP_L$. Most importantly, a pronounced inhibition of apoptosis was observed when $cFLIP_L$-expressing HaCaT KCs were exposed to activated T cells as well as their supernatants. In fact, the frequency of dead cells was comparable with "spontanous" apoptosis observed in vector control cells. However, we cannot formally exclude that other death receptor pathways, such as tumor necrosis factor, might contribute to the slight increment of the frequency of dead HaCaT KCs in cocultures with activated T cells or their supernatants. Nonetheless, these experiments show that $cFLIP_L$ is capable of inhibiting T-cell-mediated KC apoptosis *in vitro*.

Reciprocal epidermal caspase-3 cleavage and cFLIP expression in acute eczematous dermatitis

The histological hallmark of eczematous disorders consists of a marked KC pathology. During eczematous inflammation, acantholysis and spongiosis in suprabasal epidermal layers may proceed to frank vesicle formation (Figure 3a and b). As caspase-3 is the prime executioner caspase of apoptosis (Lavrik *et al.*, 2005), we next investigated the *in situ* cleavage of caspase-3 in eczematous dermatitis using an mAb that detects cleaved (as surrogate marker for activation), but not

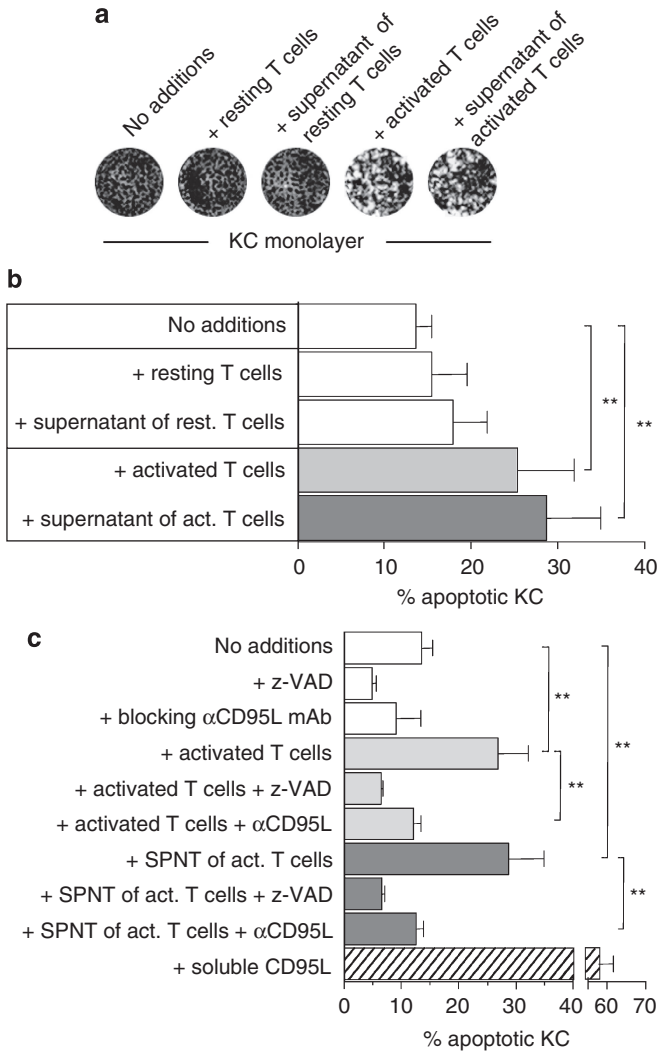

Figure 1. T-cell-mediated keratinocyte (KC) apoptosis. (**a**) Signs of HaCaT KC injury after coculture with activated T cells. Intact monolayer of HaCaT KC was cultured without additions, with resting T cells, or with their supernatant, respectively. The HaCaT monolayer after 2 days coculture with activated T cells or their supernatant is destroyed. Crystal violet staining of KC cultured in 96-well plates. (**b**) HaCaT KC apoptosis in coculture with stimulated $CD4^+$ T cells, supernatant from stimulated $CD4^+$ T cells or unstimulated controls. Cell death was analyzed as described in Materials and Methods. Data from three independent experiments performed in triplicates are shown. Error bars indicate SD. $^{**}P<0.01$, act. = activated, rest. = resting. (**c**) Role of CD95-mediated KC apoptosis in cocultures with activated T cells. HaCaT–T-cell cocultures and detection of apoptosis were performed as described in (**b**). The pan-caspase inhibitor z-VAD and a neutralizing anti-CD95L mAb (αCD95L) block T-cell-mediated HaCaT KC apoptosis. Susceptibility of HaCaT cells to CD95-mediated cell death was determined through artificial CD95 triggering (soluble CD95L). Error bars indicate SD calculated from three independent experiments performed in triplicates. $^{**}P<0.01$. SPNT = supernatant.

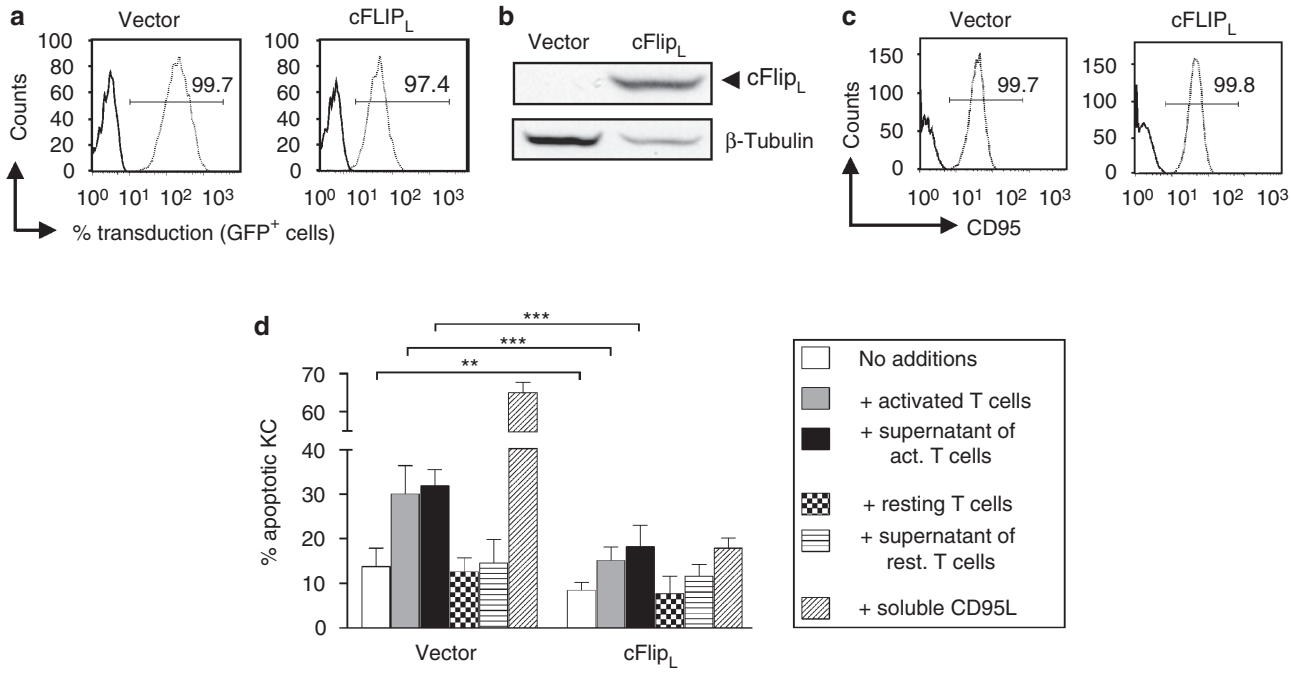

Figure 2. Retroviral infection of HaCaT keratinocytes (KCs) with cFLIP$_L$. (**a**) Green fluorescent protein expression (GFP) of HaCaT KC retrovirally transduced with bicistronic expression vectors containing the empty vector or cFLIP$_L$. (**b**) Expression of retrovirally transduced cFLIP$_L$ was confirmed by western blot analysis. β-Tubulin serves as a loading control. (**c**) Unaltered CD95 expression of empty vector or cFLIP$_L$-transduced HaCaT cells as determined by flow cytometry. (**d**) cFLIP$_L$ protects HaCaT KCs from T-cell-induced apoptosis. HaCaT cells transduced with empty vector or vector carrying cFlip$_L$ were cocultured and stimulated through CD95, as described in Figure 1b and c. The results shown represent three experiments performed in triplicates. Error bars depict SD. $^{**}P<0.01$, $^{***}P<0.001$, act. = activated, rest. = resting.

full-length, caspase-3. In all biopsy specimens tested (AD/ACD), cleaved caspase-3 was detected in single suprabasal KCs within areas of spongiosis, whereas healthy or non-lesional skin showed no signal (Figure 3c and d). In cases with spongiotic vesicle formation, single KCs positive for cleaved caspase-3 were seen in the suprabasal epidermal layers close to the vesicle (Figure 3e and f). In contrast, cFLIP expression in healthy/non-lesional skin and acute eczematous dermatitis (AD/ACD) was restricted to basal epidermal KCs (Figure 3g and h).

Apoptosis of basal keratinocytes in lichen planus

The characteristic histopathological findings of LP are the interface dermatitis, consisting of a dense subepidermal band-like lymphocytic infiltrate with vacuolization of the basal epidermal layer (Figure 4a and b). Analysis of cleaved caspase-3 in biopsy specimens of LP showed single apoptotic KC positive in the basal epidermal layer (Figure 4c and d). Interestingly, expression of antiapoptotic cFLIP in KCs at the dermal–epidermal junction was unchanged (Figure 4e and f), suggesting a mechanism of cell death different to CD95 signaling in LP. Accordingly, it has been reported that cytotoxic effector T cells expressing granzyme B are the predominant subset of lymphocytes in LP (and in other dermatoses characterized by an interface dermatitis, Wenzel and Tuting, 2008, and Figure S2). Therefore, in contrast to acute eczematous dermatitis, the perforin/granzyme pathway may bypass the antiapoptotic effect of cFLIP in LP (Wenzel *et al.*, 2006).

DISCUSSION

T-cell-mediated apoptosis of single KCs is a key feature of epidermal pathology in acute eczematous dermatitis. Interestingly, in eczema, spongiosis is predominantly located in suprabasal epidermal layers, suggesting an antiapoptotic mechanism protecting basal KCs. CD95 is slightly upregulated on KCs throughout all epidermal layers in eczematous dermatitis as compared with healthy skin (Trautmann *et al.*, 2000; Simon *et al.*, 2006). Thus, differential CD95 expression may basically account for the increased susceptibility of KCs to CD95-mediated apoptosis in eczema, but does not explain the apoptosis resistance of basal KCs. The differential expression of pro- and antiapoptotic factors, which may influence the susceptibility to CD95-mediated apoptosis, might provide an explanation for the restriction of spongiosis to suprabasal epidermal layers. Epidermal cFLIP expression was readily detected in basal KCs of healthy skin (Figure 3g), and this expression pattern was also observed in eczematous dermatitis (Figure 3h), suggesting that basal epidermal KC may be protected by cFLIP from T-cell-mediated cell death in eczematous dermatitis. This concept is supported by Bachmann *et al.* (2001) who showed that cFLIP is strongly expressed in the basal epidermal layer and squamous-cell carcinoma, thereby providing a tumor escape mechanism through an enhanced resistance to death-receptor-mediated apoptosis. However, future experiments using primary KCs with repressed cFLIP expression have to clarify the precise death-receptor-inhibitory role of cFLIP in our *in vitro* T-cell–KC coculture system.

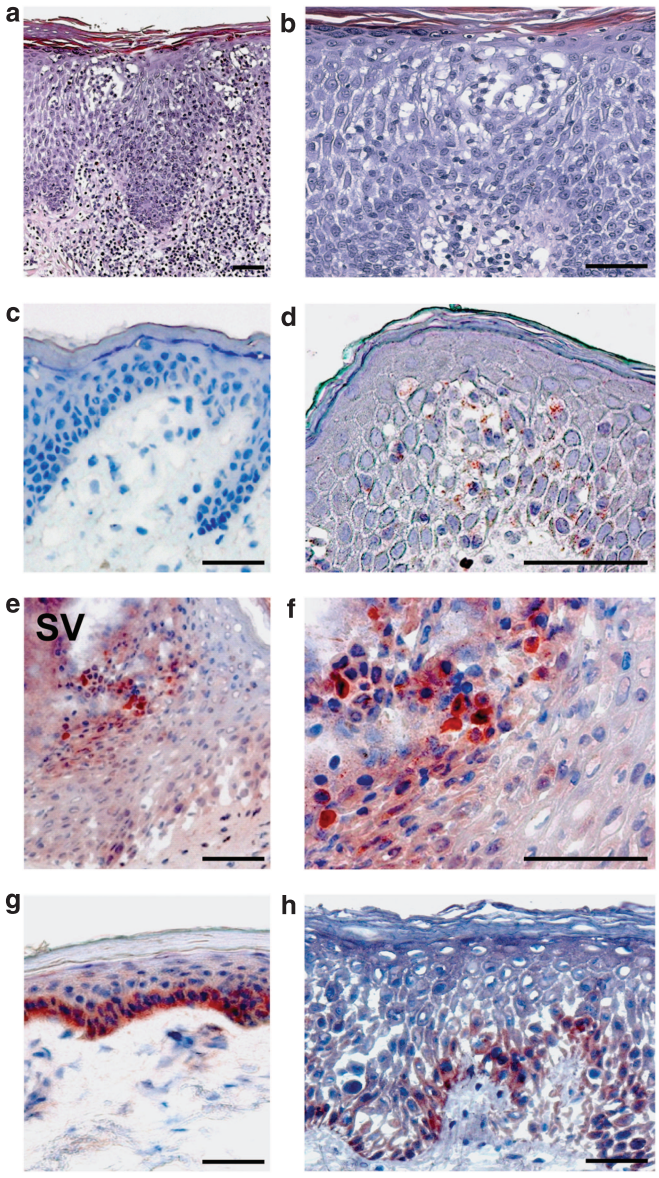

Figure 3. Features of acute eczematous dermatitis. (**a**) and (**b**) Representative histological findings of acute eczematous dermatitis (here: AD). A dense subepidermal T-cell-rich inflammatory infiltrate, and marked suprabasal epidermal akantolytic and spongiotic changes progress to vesicle formation. Hematoxylin/eosin staining. (**c**–**f**) Suprabasal caspase-3 cleavage in eczematous dermatitis. (**c**) Healthy skin as a negative control. (**d**) Eczematous skin (here: AD) with moderate spongiosis. Cleaved caspase-3 (p17) is predominantly detected in suprabasal layers. (**e**) p17 is expressed at the side and at the bottom of spongiotic vesicle (SV) formation (here: ACD). Panel (**f**) is a blow-up of panel (**e**) and highlights single KC positive for p17 expression. (**g**) Restricted expression of cFLIP to the basal layer in healthy and (**h**) lesional skin of acute eczematous dermatitis (here: ACD). Scale bars = 100 μm.

The restricted expression of cFLIP to basal epidermal KCs may be crucial for skin homeostasis/renewal. In this respect, inhibition of death-receptor-mediated apoptosis in the course of inflammatory skin diseases might ensure survival of the basal epidermal layer (including stem cells). However, under certain inflammatory settings in skin diseases involving (autoreactive) cytotoxic T cells, apoptosis of basal epidermal KC spotlights the epidermal histopathology. As shown for LP and other dermatoses going along with an interface dermatitis, cytotoxic granzyme B-expressing T cells directly attack basal epidermal KCs (Wenzel and Tuting, 2008), thereby overriding the protective role of cFLIP. It has been suggested that pathogenetically type I IFNs (IFN-α/β) play an important role in the recruitment of cytotoxic lymphocytes to the skin in interface dermatitis, whereas in eczematous dermatitis a type II IFN (IFN-γ) secreted by activated $CD4^+$ T cells is one of the major cytokines promoting skin pathology. Accordingly, using *in vitro* T-cell–KC cocultures, apoptosis of KC is inhibited in the presence of a neutralizing IFN-γ mAb, pinpointing IFN-γ as a central cytokine in eczematous skin inflammation (Trautmann *et al.*, 2000). Future studies will have to clarify if such an inhibition of type I IFNs abolishes KC pathology in LP.

The importance of the marked resistance of cFLIP-expressing HaCaT KCs against T-cell-mediated apoptosis is further stressed by recent work that showed a CD95-induced proinflammatory response of KCs (Farley *et al.*, 2006, 2008; Leverkus and Trautmann, 2006). CD95–CD95L interactions were shown to activate the transcription factor NF-κB that is critical for the CD95L-induced activation of inflammatory genes, such as IL-8. Furthermore, CD95L-stimulated secretion of EGF ligands led to the para-/autocrine activation of EGFR and the downstream activation of ERK, which was shown to mediate some of the CD95L-induced inflammatory responses. Importantly, in these studies caspase inhibitors blocked the execution of the death program, but did not suppress proinflammatory gene expression. Thus, a reasonable candidate for intracellular signal deviation upon CD95 triggering could be cFLIP (Park *et al.*, 2005). However, in contrast to the studies by Farley *et al.* (2006, 2008) using pharmacological peptide inhibitors of caspases, the physiological inhibitor cFLIP appears to inhibit death-receptor-mediated NF-κB activation and gene induction in KCs (Kreuz *et al.*, 2004; Wachter *et al.*, 2004). Nonetheless, the precise role of cFLIP as a molecular switch that allows CD95 either to mediate apoptosis or to activate the non-apoptotic mitogen-activated protein kinases and NF-κB pathways remains controversial, and may depend on the signaling strength. A recent study showed that upon moderate triggering of CD95, cFLIP was efficiently recruited to the death-inducing signaling complex, leading to the inhibition of caspase-8 activation and thereby promoting survival signals (Lavrik *et al.*, 2007). To add more complexity, studies in which the caspase-8 induced cleavage product p43 of cFLIP was ectopically expressed induced NF-κB (Kataoka and Tschopp, 2004). Furthermore, recent studies showed that cFLIP may act in concert with FADD and caspase-8 and link CD95 to non-apoptotic pathways (Park *et al.*, 2005). Thus, cFLIP seems to be an enhancer of NF-κB activation (thereby promoting survival and proliferation) in some contexts and an inhibitor in others. This argues for a highly cell-type-specific signaling capability of cFLIP. In any case, further studies have to clarify the potential role of cFLIP

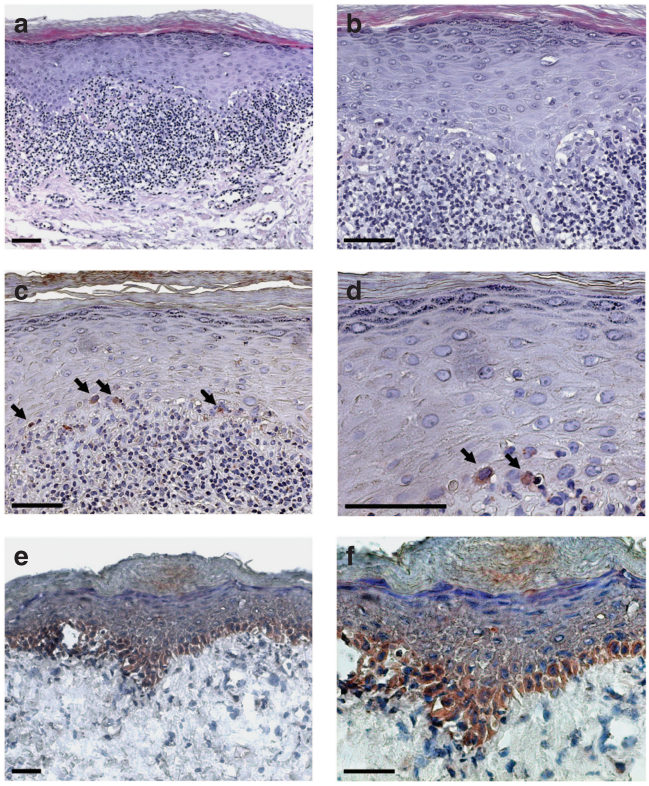

Figure 4. Features of lichen planus (LP). (**a**) and (**b**) Representative histological findings of LP. Interface dermatitis with hydropic degeneration of the basal epidermis and a dense, band-like lympho-cytic infiltrate accompanied by acanthosis, hypergranulosis, and keratosis. Note the single apoptotic KCs in the basal epidermal layer. Hematoxylin/eosin staining. (**c**) and (**d**) Single KC positive for cleaved caspase-3 (arrows) are found in the basal epidermal layer in areas of KC damage. (**e**) and (**f**) Unperturbed expression of cFLIP in the basal epidermal layer in LP. Scale bars = 100 μm.

in proinflammatory CD95-signaling in human KCs (Leverkus *et al.*, 2008).

In conclusion, we show that cFLIP protects basal KCs against T-cell-mediated apoptosis exerted in eczematous dermatitis, thereby facilitating the rapid healing of an uncomplicated eczema due to the survival of basal KCs. The knowledge of this molecular basis is pivotal in understanding the development of spongiotic pathology in eczema, and opens a future for more focused therapeutic applications.

MATERIALS AND METHODS

Subjects

Four incisional skin biopsies were taken from positive nickel patch tests (2 + according to the International Contact Dermatitis Research Group), LP and AD. Additionally, three cases of AD with acute exacerbation and three cases with LP were drawn from the files of our Department. For comparison, biopsies from non-lesional skin of three patients with positive nickel patch tests and normal skin of three healthy, non-atopic individuals were obtained. Informed consent was obtained from all subjects, and the study was approved by the Ethical Committee Board of the University of Würzburg, Germany. The study was conducted according to the Declaration of Helsinki Principles.

Antibodies and reagents

The following mAb were used for cell culture, immunohistochemistry, and flow cytometry: neutralizing anti-CD95L (NOK-2, Becton Dickinson, Heidelberg, Germany), anti-cleaved caspase-3 (5A1, New England Biolabs, Frankfurt, Germany), anti-cFLIP (G-11, Santa Cruz Biotechnology, Heidelberg, Germany), and allophycocyanin labeled anti-CD95 (DX2, Miltenyi Biotec, Bergisch Gladbach, Germany). Appropriate isotype controls were from Becton Dickinson or Dako (Hamburg, Germany).

Keratinocyte and T-cell culture

The spontaneously transformed KC line HaCaT was kindly provided by Professor Petra Boukamp (DKFZ, Heidelberg, Germany) and cultured as described (Boukamp *et al.*, 1988). Mononuclear cells were isolated by Ficoll (Linaris, Wertheim, Germany) density gradient centrifugation of peripheral venous blood of healthy volunteers. $CD4^+$ cells were negatively selected using the MACS system ($CD4^+$ isolation Kit II) according to the manufacturer's instructions (Miltenyi Biotec). Purified $CD4^+$ cells resuspended in RPMI 1640 (supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin; all from PAN Biotech, Aidenbach, Germany) were costimulated (10^6/ml) in six-well dishes with a combination of soluble anti-CD2 (4B2 and 6G4, each 0.5 μg ml^{-1}), anti-CD3 (UCHT1, 1 μg ml^{-1}), and anti-CD28 (CD28.2, 1 μg ml^{-1}) mAb (Trautmann *et al.*, 2000). After 24 hours, cells were harvested and washed twice with medium before the initiation of T-cell–KC cocultures. In parallel, supernatants were retained and cleared by centrifugation.

Keratinocyte–T-cell cocultures

For coculture experiments, HaCaT cells were cultured in six-well plates in the presence or absence of activated $CD4^+$ T cells at a ratio of 3:1. For studying the effects of T cells on KCs, T-cell supernatants were collected, diluted 1:2 with supplemented RPMI 1640, and added to HaCaT cells in six-well plates.

Retroviral transduction

The pCFG5-IEGZ retroviral vector containing complementary DNA inserts of $cFLIP_L$ (Geserick *et al.*, 2008) was used for infection of HaCaT KCs. Briefly, the amphotrophic producer cell line φNX was transfected with 10 μg of the retroviral vectors by calcium phosphate precipitation. Cell-culture supernatants containing viral particles were generated by incubation of producer cells with HaCaT medium (DMEM containing 10% fetal calf serum) overnight, as described (Diessenbacher *et al.*, 2008). Following filtration, the culture supernatant was added to HaCaT cells seeded in six-well plates 24 hours earlier in the presence of 1 μg ml^{-1} of polybrene. HaCaT cells were centrifuged for 3 hours at 32 °C, and viral particle containing the supernatant was subsequently replaced by fresh medium. After 10–14 days, the recovery of bulk-infected cultures in the presence of Zeocin (300 μg ml^{-1}) was carried out. FACS analysis for green fluorescent protein expression and western blot analysis (Figure 2a and b) was performed on polyclonal expanded cells to confirm ectopic $cFLIP_L$ expression.

Induction, inhibition, and detection of apoptosis

Sensitivity of HaCaT KC to CD95-mediated apoptosis was assessed by incubation with a soluble Flag-tagged fusion protein of CD95L

(0.1 μg ml^{-1} Axxora, Lörrach, Germany), which was crosslinked through an anti-Flag mAb (1 μg ml^{-1}; Enhancer, Axxora). Inhibition of CD95-induced apoptosis was tested with preincubation of supernatants and activated T cells with a neutralizing anti-CD95L mAb. For the protease inhibition assay, cells were preincubated for 1 hour with 40 μM of z-VAD-fmk (Z-Val-Ala-Asp-fluoromethyl ketone, Sigma-Aldrich, Schnelldorf, Germany). After 48 hours incubation, KCs were harvested and resuspended in annexin-V-binding buffer (0.01 M HEPES pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$) containing FITC-labeled annexin V (Becton Dickinson) and 7-actinomycin D (Sigma-Aldrich). After 15 minutes at 4 °C in the dark, the samples were diluted in buffer and immediately analyzed in a FACSCanto flow cytometer (Becton Dickinson). In the flow cytometry setting, HaCaT and T cells were gated according to forward and side scatter. Crystal violet staining of attached and viable cells was performed 2 days after incubation with activated T cells and their supernatant, respectively, in 96-well plates.

Immunohistology

The tissue samples were placed in Tissue-Tek OCT compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) and stored at −140 °C. Cryostat sections (4–5 μm) were prepared on silanized slides (Superfrost plus, Langenbrinck, Teningen, Germany). After air drying, the sections were fixed in acetone (for detection of cFLIP) or 10% buffered formalin solution (for detection of cleaved caspase-3) for 10 minutes at 4 °C. The sections were then incubated with mouse anti-cFLIP mAb (10 μg/ml) or rabbit anti-cleaved caspase-3 mAb (0.5 μg/ml) at 4 °C overnight. This was followed by incubation with a combination of biotin-conjugated goat-anti-mouse/goat-anti-rabbit IgG (Dako), and subsequently streptavidin-conjugated horseradish-peroxidase (Dako) at room temperature for 1 hour. Incubation with the peroxidase-specific substrate 3-amino-9-ethylcarbazole (Sigma-Aldrich) was used for visualization, with hematoxylin counterstaining.

Alternatively, detection of cleaved caspase-3 was evaluated on paraffin-embedded specimens of AD (3 ×) or LP (3 ×) drawn from the files of our Department. Briefly, 5 μm paraffin sections were dewaxed and rehydrated with xylene and graded alcohols, followed by unmasking of antigen epitopes by boiling in citrate buffer pH 6.0 (Dako) for 10 minutes. After cooling to room temperature, endogenous peroxidase was quenched with hydrogen peroxide. Thereafter, cleaved caspase-3 was detected as described above. For control purposes, the primary mAb was replaced by an irrelevant isotype-matched mAb or rabbit IgG, which consistently yielded negative results. The stained sections were evaluated by light microscopy (Axiophot, Carl Zeiss, Jena, Germany).

Western blot

Cell lysates were prepared essentially as described before (Leverkus *et al.*, 2003). Lysates of 5×10^5 cells per lane were separated by SDS-PAGE on 4–12% NuPAGE Bis-Tris gradient gels (Invitrogen, Karlsruhe, Germany) and transferred to nitrocellulose membranes. Subsequently,
membranes were probed with mAb to cFLIP (NF-6, Axxora) or to β-tubulin (Sigma-Aldrich), as indicated followed by the appropriate secondary antibody-peroxidase conjugate, and developed using the ECL detection system (Amersham Biosciences, Freiburg, Germany).

Statistical analyses

Where indicated, data were subjected to unpaired *t*-test (GraphPad Prism 5.0; GraphPad). Values of $P < 0.05$ were considered to be significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Supplementary Materials and Methods

Figure S1. Differential expression of cFLIP in transduced HaCaT and primary keratinocytes (nKC).

Figure S2. T-cell subpopulations in eczematous dermatitis and lichen planus.

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